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Detection and Comparison of DNA Adducts after *in Vitro* and *in Vivo* Diesel Emission Exposures

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Development of methods to evaluate certain classes of polycyclic aromatic compounds (PAC) detected in complex mixtures to which humans are exposed would greatly improve the diagnostic potential of ^{32}P-postlabeling analysis. Identification of DNA adduct patterns or specific exposure-related marker adducts would strengthen associations between observed DNA adducts and exposures to different environmental pollutants (e.g., kerosene, cigarette smoke, coke oven, and diesel). We have compared diesel-modified DNA adduct patterns in various *in vitro* and *in vivo* rodent model systems and compared them to DNA reactive oxidative and reductive metabolites of 1-nitropyrene. The formation of nitrated polycyclic aromatic hydrocarbon (nitrated PAH) DNA adducts, derived from the metabolism of diesel extract constituents, was enhanced relative to other PAH-derived DNA adducts via xanthine oxidase-catalyzed nitroreduction. These adducts were detectable only by the butanol extraction version of the postlabeling analysis. Five major DNA adducts were detected in human lymphocytes treated *in vitro* with diesel extract. A major adduct detected in human lymphocytes treated *in vitro* with diesel extract co-migrated with a major adduct detected in lymphocyte DNA treated with benzo[a]pyrene (BaP) alone. Other adducts that co-migrated with the major BaP-derived adducts were detected in skin and lung DNA isolated from rodents topically treated with (50 mg) diesel extract and the major adduct detected in calf thymus DNA treated with rat liver S9 and diesel particle extract. Postlabeling of lung DNA isolated from rodents exposed via lung inhalation for 24 months to diesel combustion emissions resulted in the formation of a major nuclease-P1-sensitive DNA adduct that did not co-migrate with the major BaP-diol epoxide adduct. Based on its sensitivity to nuclease- P1, this adduct may be an *N*-substituted aryl adduct. Marker adducts detected in the various test systems presented here will assist in characterizing nuclease-P1-sensitive nitrated PAH adducts in humans.

Introduction

Nitrated polycyclic aromatic hydrocarbons (nitrated PAH) are emitted from several combustion emission processes (e.g., diesel). They are also formed in ambient air through atmospheric reaction (*1*). Thus, there is interest in studying the role nitrated PAH play in human cancer. Nitrated PAH are present in conjunction with other PAH, therefore it is difficult to ascertain the importance of nitrated-PAH relative to other PAH in the cancer process. Refinement of DNA adduct methods (e.g., ^{32}P-postlabeling analysis) to provide information about the identity of nitrated PAH DNA reactive intermediates relative to other PAH intermediates would substantially improve the diagnostic potential of ^{32}P-postlabeling methods. In this paper, we describe the diesel-derived DNA adducts resulting from several *in vitro* and *in vivo* model systems. The detection of specific adducts that are unique to diesel exposure will be useful when analyzing DNA adducts detected in human tissues and cells exposed to complex emission sources containing nitrated PAH.

Diesel combustion emissions or particle extracts of these emissions are used as a model because these emissions contain relatively high concentrations of nitrated PAH compared to other PAH (e.g., 1-nitropyrene; 1-NP). Calf thymus (CT) DNA adduct patterns observed after reductive or combinations of reductive and oxidative metabolism of 1-NP are compared to DNA adduct patterns resulting from modifying CT DNA and human lymphoctyes *in vitro* with diesel particle extracts. DNA adduct patterns resulting from topical application of diesel extract or inhalation of diesel combustion emissions are compared.

We also present methods that enhance the formation of nitrated PAH-derived adducts from the array of other PAH reactive metabolites through xanthine oxidase-mediated nitroreduction of the nitrated PAH constituents in the diesel organic extracts. Based on the marked differences in sensitivity for the detection of *N*-substituted arylamine adducts for the two most commonly

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used versions of the ^{32}P-postlabeling methodology (*2,3*), we have tentatively identified a number of *N*-substituted arylamine adducts.

Materials and Methods

In Vitro Assays

Isolation of Reductive/Oxidative Metabolites of 1-NP. 1-Nitropyrene (20 μM) was incubated for 1.5 hr at 37 °C in the presence of xanthine oxidase (XO) (0.5 units/mL) and hypoxanthine 0.6 mg/mL in an anaerobic chamber. To retain the reductive metabolites generated by the xanthine oxidase-catalyzed reduction of 1-NP, the incubate was passed through two primed (10 mL of MeOH and 10 mL water) C-18 Sep-Pak cartridges. The metabolites were eluted from the column using 10 mL of HPLC-grade methanol. The extract containing the metabolites was evaporated to dryness under a stream of nitrogen. The dried extract was redissolved in a 16 mM phosphate buffer (pH 7.41) and incubated in a shaking water bath under aerobic conditions for 1.5 hr at 37 °C in the presence of 1 mL of a 2-mg/mL CT DNA solution and rat liver S9 (0.5 mg protein/mL). The rat liver S9 was prepared as previously reported (*4*). The reaction was terminated by quick freezing the reaction mixture in liquid nitrogen. Samples were stored at -70 °C before DNA extraction.

Formation of DNA Reactive Metabolites (Reductive) of 1-NP. To catalyze the nitroreduction of 1-NP (20 μM), CT DNA (1 mg/mL), XO (0.5 units/mL), and hypoxanthine (0.6 mg/mL) were incubated under anaerobic conditions, in a total reaction volume of 2 mL. The reaction was terminated by the addition of 1 volume of chloroform:isoamyl-alcohol:phenol (24:1:25).

Diesel Modified-Calf Thymus DNA. Preparation of the diesel particle extracts used in the study have been previously described (*5*). To obtain oxidative metabolites, CT DNA (1 mg of a 2-mg/mL DNA solution) was modified with diesel combustion particle extract (100 μg/mL), and rat liver S9 (0.5 mg/mL) under aerobic conditions for 1.5 hr at 37 °C. Reductive metabolites were generated by adding XO (0.5 units/mL) and hypoxanthine (0.6 mg/mL) under anaerobic conditions for 1.5 hr at 37 °C in the presence of 1 mg of CT DNA.

In Vivo Treatments

Skin Painting. Female C-57 mice were topically treated with 50 mg diesel extract as previously described (*6*). Twenty-four hours after the last exposure, the mice were sacrificed and skin removed and stored at -70 °C until subsequent DNA isolation.

Inhalation. Cdl(WI)Br rats were exposed to diesel combustion emissions containing 7.5 mg/m^3 soot for a period of 24 months. Tissues were kindly provided by U. Heinrich of The Fraunhofer-Institute of Toxicology and Pharmacology, Hannover, Germany.

DNA Extraction and ^{32}P-Postlabeling Analysis. DNA was isolated from CT DNA and rodent tissue essentially as described by Gupta (*7*). Human lymphocyte DNA was isolated as described by Maniatis et al. (*8*). Twelve micrograms of DNA was digested to mononucleotides at 37 °C for 3.5 hr with micrococcal endonuclease and spleen phosphodiesterase (*6*). Selected samples were divided into two aliquots and DNA adducts were enriched from one aliquot by the butanol extraction version of the ^{32}P-postlabeling assay (*9*) and the other by the nuclease P1 treatment essentially as previously described (*10*). Intensifying screen-enhanced autoradiography at -70 °C was used to detect the presence of radiolabeled adducts on the thin layer chromatography plates. Exposure times for autoradiograms are shown in the figure legends.

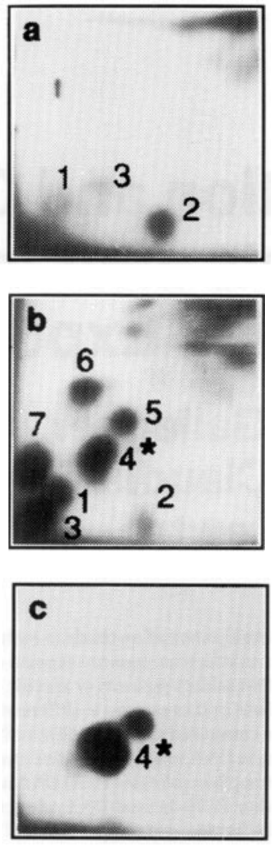

FIGURE 1. ^{32}P-postlabeled DNA isolated from human peripheral blood lymphcoytes. (*a*) Background adducts as detected in lymphocyte DNA isolated from a nonsmoking individual following treatment *in vitro* with dimethylsulfoxide. (*b*) ^{32}P-postlabeled DNA isolated from human lymphocytes incubated for 18 hr with 1 μg/mL diesel particle extracts. (*c*) Human lymphocyte DNA after treatment *in vitro* with 1 μM BaP. Exposure times for the autoradiograms were 64 hr (panels *a* and *b*) and 18 hr (panel *c*). For purposes of clarity, all DNA adducts which migrate with the major BaP-derived DNA adduct are identified (*).

Results and Discussion

It is often difficult to correlate DNA adduct levels detected in human tissue and cells with occupational or environmental exposure due to the differences in interindividual responses to various carcinogens. Moreover, the exposures under investigation are typically confounded by unrelated exposures.

^{32}P-postlabeling methods are commonly used to assess the overall DNA adduct burden as a result of exposure to complex air pollution mixtures. With respect to DNA adduct identification, improvement in ^{32}P-postlabeling methods will be necessary if advances are to be made with respect to distinguishing the important PAH in the mixture that play a key role in the cancer process.

White blood cells (WBC) have been proposed to be useful surrogates for monitoring exposures in selected workers. Human

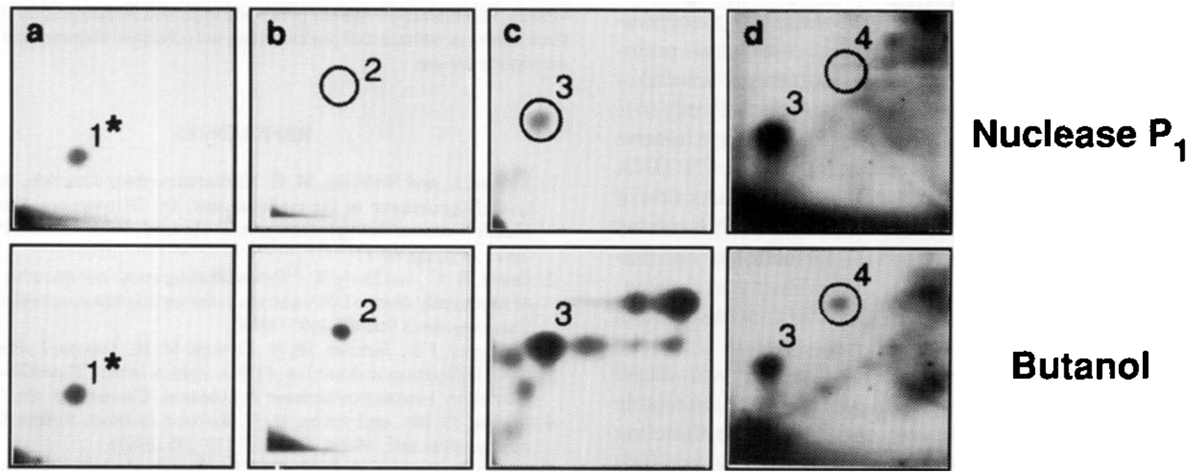

FIGURE 2. DNA adducts patterns for calf thymus DNA following *in vitro* rat liver S9-mediated metabolism (*a*) or xanthine oxidase-catalyzed nitroreduction of diesel particle extract (*b*). (*c*) DNA adducts resulting from xanthine oxidase-mediated nitroreduction of 1-nitropyrene. (*d*) DNA adducts resulting from reductive/oxidative metabolism of 1-nitropyrene as described in the Materials and Methods. Control DNA samples in which S9 and/or xanthine oxidase was added to calf thymus DNA were virtually devoid of radioactivity. Exposure times for the autoradioagrams were 6 hr (*a*, *b*) and 24 hr (*c*,*d*). (*) DNA adducts that migrate with the major BaP-derived adduct.

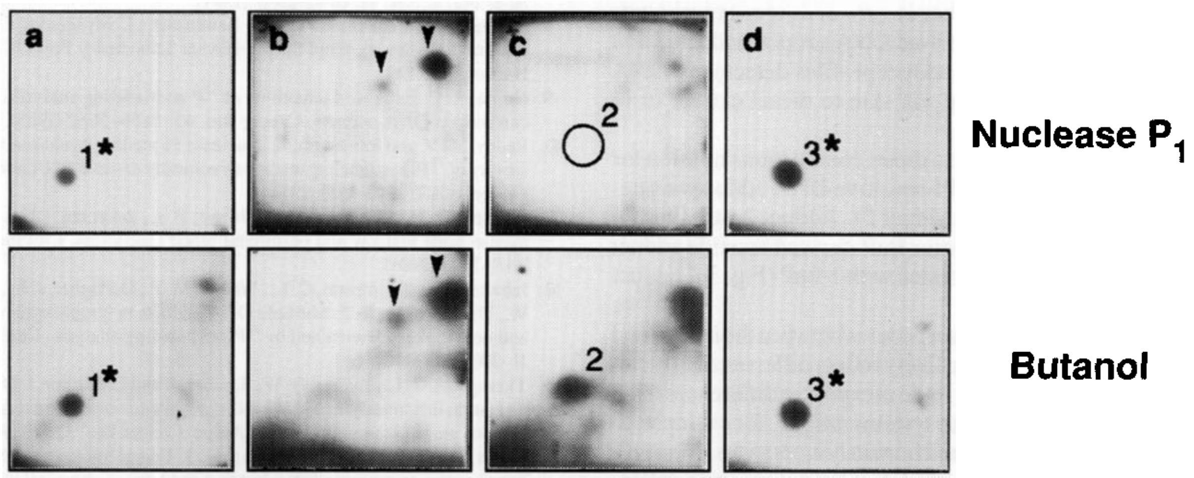

FIGURE 3. Comparison of skin DNA adducts following skin application of diesel extract and 1 μM BaP (*a*,*d*) with lung DNA adducts formed following inhalation of sham-exposed (*b*) and diesel emission-exposed rodents (*c*). Exposure times were 6 hr (*a*), 5 hr (*b*,*c*), and 24 hr (*d*). (*) DNA adducts co-migrating with the major BaP-derived DNA adduct.

lymphocytes were treated *in vitro* with 1 μg/mL diesel particle extract or dimethylsulfoxide (DMSO). Background DNA adducts, of unknown origin, were observed in the DMSO-treated human lymphocytes (Fig. 1*a*, adducts 1–3). The presence of background levels of adducts in blood cell DNA is consistent with studies where DNA adducts were observed in nonsmokers with no known occupational exposures (*11*,*12*). Five diesel-derived DNA adducts were reproducibly detected in lymphocyte DNA (Fig. 1*b*, adducts 3–7). One major DNA adduct detected in diesel-treated human lymphocyte DNA (Fig. 1*b*, adduct 4) co-migrated with the major BaP-derived DNA adduct detected in human lymphocytes treated with 1 μM BaP (Fig. 1*c*, adduct 4). Previous studies indicate that human lymphocytes can metabolize single chemicals as well as complex air pollution particle extracts (*13*,*14*). Future studies to evaluate the differences in labeling efficiency by comparing DNA adduct patterns in the nuclease P1 and butanol extraction versions of the postlabeling assay may reveal evidence for the presence of additional *N*-substituted arylamine adducts resulting from the nitroreduction of nitrated PAH present in diesel extracts. 1,6-Dinitropyrene, for example, when administered via lung implantation has been shown to be metabolically activated by nitroreduction to form DNA adducts in spleen lymphocytes (*15*).

In vitro assays using CT DNA and rat liver S9 treated with diesel particle extract resulted in the formation of one major DNA adduct (Fig. 2*a*, adduct 1) detectable by the butanol and nuclease P1 version of the assay. Adduct 1 also co-migrated with the major BaP-diol epoxide DNA adduct detected in skin DNA (Fig. 3*d*, adduct 3) and with the major DNA adduct detected in

DNA isolated from rodents topically treated with 50 mg diesel particle extract (Fig. 3*a*, adduct 1). Based on the low concentration of BaP in diesel extracts, relative to other complex air pollution mixtures that we have analyzed in this laboratory, it is not likely that the diesel-derived DNA adducts arise from BaP only (*6*).

The formation of nitrated PAH-derived DNA adducts relative to other PAH-derived DNA adducts detected in our CT DNA model systems was achieved by XO-mediated nitroreduction of nitrated PAH constituents in the diesel extract. CT DNA treated with diesel extract and XO resulted in the formation of one major DNA adduct (Fig. 2*b*, adduct 2). This adduct was chromatographically distinct from the major DNA adduct observed in the rat liver S9-treated incubations (Fig. 2*a*, adduct 1) and the major adduct detected in rodent skin DNA treated with diesel particle extract (Fig. 3*a*, adduct 1). Adduct no. 2 was detectable only with the butanol extraction version of the ^{32}P-postlabeling assay (Fig. 2*b*). Based on its nuclease P1 sensitivity and similarity in chromatographic properties compared to a major 1-NP-derived adduct (Fig. 2*c*, adduct 7), adduct 2 has been tentatively identified as an *N*-substituted arylamine adduct.

Two adducts with different sensitivity to nuclease P1 monophosphatase activity were observed (Fig 2*d*, adducts 3,4) when 1-NP was metabolized to reductive and oxidative metabolites in the presence of CT DNA. Adduct 4 was unique in as much as it has not been detected in any other *in vitro* or *in vivo* experiments employed in our study. 1-NP-derived DNA adduct (adducts 3 and 4) were not observed in DNA adduct profiles detected in DNA isolated from rodents exposed via skin to diesel extract or to diesel particles via inhalation.

When rodents were exposed to diesel combustion emission for 24 months, a major nuclease-P1-sensitive DNA adduct was detected in lung DNA (Fig. 3*c*, adduct 2). Adduct 2 was slightly more polar compared to the major BaP-derived epoxide adduct detected in rodent skin DNA treated with 1-BaP (Fig. 3*d*, adduct 3).

The differences in DNA adduct patterns between the *in vitro* and *in vivo* (skin and lung) exposure likely reflect differences in metabolic pathways as influenced by route of exposure differences (skin versus lung) or specific enzyme systems unique, for example, to rat liver S9, xanthine oxidase, and human lymphocytes. Many nitrated PAH are tumorigenic via inhalation but not via skin application (*16,17*), and this is consistent with differences in the type of adducts that we observed for the two exposure routes. These observations emphasize the potential importance of nitrated PAH found in diesel emissions or in air-containing nitrated aromatics. In fact, we have preliminary evidence of such nitrated PAH (or other *N*-substituted arylamine adducts in human autopsy lung samples (*18*). Future studies will focus on other combinations of metabolic pathways to further characterize the nature of DNA adducts as detected in *in vivo* experimental systems.

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